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14. ABSTRACT

The core of my hypothesis is that Steroid receptor RNA activator protein, a newly discovered protein from a once thought non-coding RNA, is involved in the modulation of the estrogen signaling pathway. The steroid receptor RNA activator (SRA) differs from all previously characterized co-activators as it is currently the first molecule potentially active at both the RNA and protein levels. Today, all functional studies on the human SRA concentrate on its RNA function and ignore the existence of the protein. We therefore believe there is an urgent need to analyze the function of the SRA protein (SRAP). The aim of the first year of the project was to first determine if SRAP affects the expression of ER target genes such as pS2 and PR. Additional aims for the first year were also to determine the effect of SRAP expression and the growth rate and invasive properties of MCF-7 cells over-expressing SRAP. In the first year, I have shown that the expression of pS2 is unaffected by SRAP. However PR is differentially expressed in the cells over-expressing SRAP. I have also determined that MCF-7 cell lines stably expressing SRAP are not a suitable model to analyze proliferation and invasion differences. In the interim, I have successfully generated the plasmids necessary to investigate SRA RNA and protein function independently. Through mass spectrometric analysis of proteins co-immunoprecipitated with SRAP, I have identified 69 possible SRAP interacting proteins.

15. Subject Terms - Cell biology, molecular biology, Estrogen receptor, Steroid receptor RNA activator

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Introduction

The core of my hypothesis is that Steroid receptor RNA activator protein, a newly discovered protein from a once thought non-coding RNA, is involved in the modulation of the estrogen signaling pathway. The steroid receptor RNA activator (SRA) was discovered in 1999 and was shown to differ from all previously characterized co-activators as it was demonstrated to function as a RNA rather than a protein molecule. We have however demonstrated that this once thought non-coding RNA encodes a well conserved protein (SRAP).

To investigate the possible implications of SRAP expression on breast cancer progression, we examined by Western blot analysis ER positive primary breast from patients subsequently treated with Tamoxifen. Our results showed that patients whose primary tumors were positive for SRAP expression had a significant lower likelihood to die from recurrent disease than SRAP negative patients. These results prompt us to investigate the possible effects of SRAP on the estrogen receptor signaling pathway. MCF-7 cells stably over-expressing SRAP have a significant decreased sensitivity to estradiol but no additional sensitivity to Tamoxifen in an ER luciferase reporter assay. A possible role of SRA in the modulation of the ER signaling pathway is intriguing as SRA is, to our knowledge, the first molecule potentially active at both the RNA and protein levels. Today, all functional studies on the human SRA concentrate on its RNA function and ignore the existence of the protein. We therefore believe there is an urgent need to analyze the function of the SRA protein (SRAP).

The aim of the first year of the project was to first determine if SRAP affects the expression of ER target genes such as pS2 and PR. Additional aims for the first year were also to determine the effect of SRAP expression and the growth rate and invasive properties of MCF-7 cells over-expressing SRAP.

In the first year, I have shown that the expression of pS2 is unaffected by SRAP. However PR is differentially expressed in the cells over-expressing SRAP. I have also determined that MCF-7 cell line stably transfected with an empty plasmid and MCF-7 cell lines stably expressing SRAP are not a suitable model to analyze proliferation and invasion differences. I have found that although generated from the same parental cell line, our SRAP expressing and control cell lines have evolved and differentiated from one another in such a way that even the growth rate of the two control cell lines is radically different. I therefore believe that an inducible SRAP MCF-7 model will be a better model to study proliferation and invasion. In the interim, I have directly pursued specific aims of task 2a and task 3 a) proposed in the statement of work (SOW) for months 9-15 and months 22-30. I have therefore successfully generated the plasmids expressing functional SRA RNA and protein independently. Through mass spectrometric analysis of proteins co-immunoprecipitated with SRAP, I have identified 69 possible SRAP interacting proteins.

Body

Task 1: To characterize the cellular and biological effects of SRA and SRAP in MCF-7 cells

a) To analyze the expression of ER target genes PR and pS2 in MCF-7 cells stably over-expressing SRAP.

To monitor pS2 and PR expression, we used MCF-7 cells lines stably transfected with either a empty vector or a expression vector containing a coding SRA cDNA. These cell lines comprise two control lines and two lines where we observed transfected SRAP over-expression by Western blot analysis. Detailed experimental procedures and descriptions regarding these cell lines are in appendix 1. We had found that the MCF-7 cells stably over-expressing SRAP have a significant decreased sensitivity to estradiol but no additional sensitivity to Tamoxifen in an ER luciferase reporter assay.

We observed that the estradiol mediated regulation of the two ER target genes studied (PR and pS2) was dissimilar in the SRAP-V5 over-expressing and control cells. In fact, while the estradiol mediated induction of the PR was evidently distinct between the SRAP over-expressing and control cell lines, no difference in pS2 induction was observed between the cells. (See appendix 1 for detailed experimental procedures as well as detailed observations and interpretations)

b) To determine the effect of SRA expression on the growth rate of the MCF-7 cells over-expressing SRAP.

We had initiated our experiments with the same MCF-7 cells lines stably expressing SRAP mentioned above. Two of these cells lines are positive and two are negative for transfected SRAP expression. However, our preliminary data have shown us that our stably transfected MCF-7 cells is not an appropriate model to compare growth rate between control and SRA over-expressing cells. Indeed, we have found that the growth rates of the different cell lines even in the absence of hormonal treatment are quite different. In fact, even the growth rates of the two control cell lines (not expressing transfected SRAP) are significantly different from one another. We therefore suspect that a stably transfected cell model is therefore not the appropriate tool to investigate the effect of SRAP expression on proliferation. Although, these cell lines were generated from the same parental line, we suspect that these cell lines, through long term isolation, have evolved and thus have different metabolisms which result in distinct proliferation rates. The stably transfected MCF-7 cells are therefore not an appropriate model for analyzing the effect of SRAP expression on growth rates. We thus propose to conduct proliferation assays in inducible SRAP MCF-cell lines. We believe that an inducible cell line is a better model for our studies since the induction of SRAP will not require long term isolation of cell populations.

c) To determine invasive properties of cells over-expressing SRA

We have decided to conduct the cell invasion assays on MCF-7 cells where we can induce SRA expression for the same reasons as the ones stated for the proliferation assays. (see task 1b)

Task 2. To characterize the respective actions of SRA RNA and protein on ER activity

a) To develop plasmids that express functional SRA RNA and protein independently.

We have successfully generated the four constructs that will allow us to characterize the respective actions of SRA RNA and protein. These four constructs are:

1. **SRARNA** that contain the full SRA sequence but with the first two ATG codons mutated. The corresponding RNA can not be translated (as it does not contain the correct initiating methionine codons) but will be functional at the RNA level (presence of an intact core region).
2. **SRAPROT** contains the full SRA cDNA but with the two series of silent mutations SDM1 and SDM7 previously shown to abolish the SRA RNA co-activator function. The corresponding RNA can not be functional but can encode the wild type SRA protein.
3. **SRARNAPROT** contains the full wild type SRA sequence. The corresponding RNA is translatable and fully functional.
4. **SRANEG** contains the full SRA sequence but with the first two ATG codons mutated and with the two series of silent mutation SDM1 and SDM7. The corresponding RNA should be non-functional and non-translatable.

All four vectors have been sequenced to confirm the incorporation of the appropriate mutations.

b) To perform assays to determine the respective functions of SRA RNA and protein

Now that we have generated the constructs, we will, as expected, proceed to perform the functional assay this year. The results of this assay will allow us to determine the respective functions of SRA RNA and protein on the estrogen-signalling pathway.

Task 3. To identify proteins interacting with SRAP

a) To perform co-immunoprecipitation assays and 2-D gel analysis for identifying protein interacting with SRA proteins.

Although, we had proposed to perform this task only in the third funding year, we decided to undertake this task in the first year. There are two main reasons for this deviation from the original statement of work. As mentioned above, we were not able to accomplish the first task stated since our proposed model of study (MCF-7 cell lines stably expressing SRAP) revealed itself to be inappropriate for the task envisioned. While we are establishing an alternative more suitable model, we therefore decided to dedicate ourselves to this third task. Secondly, we also thought that if we can determine SRAP

interacting proteins we will have additional insights in the mechanisms of SRAP action that could facilitate the interpretations of the results from task 1 and task 2.

In order to identify SRAP interacting proteins, we first immunoprecipitated V5 tagged SRAP from the total cell lysates of MCF-7 cells over-expressing SRAP-V5. As a control we used V5 peptides to compete out the precipitation of SRAP-V5 (figure 1 appendix 3). We subsequently analyzed both SRAP-V5 co-immunoprecipitated and control samples by mass spectrometric analysis. We have identified 69 proteins specifically interacting with SRAP. Out of these 69 proteins we have found that 35 are involved in general protein metabolism such as protein biosynthesis, degradation (proteosomal proteins), and chaperone proteins (figure 2, appendix 3). These proteins are thus most likely involved in the general synthesis of SRA proteins. We are therefore focusing our analysis on the remaining 32 proteins as they have other functions and thus could give us insights into SRAP mechanisms of action. Interestingly, 9 out of these 32 proteins are known to be involved in transcriptional regulation. Furthermore 2 are known to be associated with estrogen receptors (figure 3 appendix 3).

b) To validate the SRAP-protein interaction results obtained in task 3a.

We will conduct GST pull down and co-immunoprecipitation assays to validate our results.

Key research accomplishments:

PS2 expression is the same in control MCF-7 cells and cells over-expressing SRAP. PR expression is higher in cells over-expressing SRAP upon estradiol treatment.

MCF-7 cell lines stably expressing SRAP are not a suitable model to study the effect of SRAP expression on cell proliferation and invasion.

We have identified 69 candidate SRAP interacting proteins. 32 of these proteins have functions other than general protein metabolism. 9 out of these 32 proteins are known to be involved in transcriptional regulation.

In progress:

Generation of a inducible SRAP cell line to study the effect of SRAP expression on proliferation and invasion rates.

Functions assays using constructs generated in aim 2 a, to determine the individual SRA RNA and protein functions

Reportable outcomes:

a) Publications:

Chooniedass-Kothari S, Hamedani MK, Troup S, Hube F, Leygue E (2005): The steroid receptor RNA activator protein is expressed in breast tumor tissues. Int.J.Cancer. (see appendix 1)

b) Poster presentation:

Chooniedass-Kothari S, Wang X, M Hamedani, S Caracossa , S Jalaguier , V Cavailles, E Leygue. The Steroid Receptor RNA Activator Protein (SRAP) possesses an intrinsic transcription repressive activity. Keystone Symposia: Nuclear Receptors: Steroid Sisters. Banff Alberta. March 18 - 23, 2006. (See appendix 2)

Conclusion

During the first year of my project, I have shown that the expression of PR, an ER target gene is higher in SRAP over-expressing cells while the expression of pS2 another ER target gene remains the same in SRAP overexpressing and control cells. We therefore believe that SRAP modulated ER transcriptional activity selectively depending on the target genes. I have also determined that MCF-7 cells stably expressing SRAP are not a suitable model for investigating cellular processes such as proliferation and invasion. Although this has brought a set back in my progress of task 1, I believe that by conducting these experiments I have gained significant knowledge which will benefit my future research work. While establishing a more suitable model to study the effect of SRAP expression on proliferation and invasion properties, I have successfully identified several SRAP interacting partners. It is interesting to note that several of these proteins are involved in transcriptional regulation. Two of these proteins are known to interact with the estrogen receptor. These results thus sustain our initial hypothesis that SRAP is involved in the modulation of estrogen receptor signaling pathway.

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Appendix 1

a) Title

The steroid receptor RNA activator protein is expressed in breast tumor tissues

b) Authors

Shilpa Chooniedass-Kothari, Mohammad Hamedani, Sandy Troup, and Etienne Leygue[2]

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c) Running title

SRAP in human breast tumors.

d) Key words

SRA, steroid receptor co-activator, human breast tumor, *SRAP*.

e) Footnotes

1 This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI # 12326). E. L. is a recipient of a USAMRMC Career Development Award (DAMD17-01-1-0308) and S C-K is a recipient of a USAMRMC Pre-Doctoral training Award.

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3 The abbreviations used are: *SRA*, steroid receptor RNA activator; *SRAP*, protein encoded by the steroid receptor RNA activator; ER, estrogen receptor; PR, progesterone receptor; *GAPDH*,

glyceraldehyde-3-phosphate dehydrogenase; TFF1, trefoil factor 1.

f) Text and References

Abstract

The steroid receptor RNA activator (SRA) has originally been described as the first functional non-coding RNA able to specifically co-activate the activity of steroid receptors. We have recently demonstrated the existence in breast cancer cell lines of new SRA isoforms, which as opposed to the first cloned SRA RNA, encode for a 236 amino-acid long protein, SRAP. To date, all data on human SRA focus only on its non-coding RNA form. In order to investigate the possible implications of the coding SRA RNA and SRAP expression on breast cancer progression, we examined by Western blot analysis 74 primary breast tumors of patients subsequently treated with Tamoxifen. Our results showed that patients whose primary tumors were positive for SRAP expression (n=24) had a significant (Kaplan Meir survival curve, $p = 0.047$) lower likelihood to die from recurrent disease than SRAP negative patients (n=50). These results prompt us to investigate the possible effects of SRAP on the estrogen receptor signaling pathway. We generated two cell lines, SRAP-V5-High.A and SRAP-V5-High.B, by stably over-expressing SRAP protein in the estrogen receptor positive MCF-7 breast cancer cell line. Transient transfection experiments, performed using a luciferase reporter gene under the control of an estrogen responsive element ERE, revealed a decreased sensitivity to estradiol but no additional sensitivity to Tamoxifen in SRAP over-expressing cells. Furthermore, we also observed a faster PR induction upon estradiol stimulation in the cells over-expressing SRAP. Overall, our data suggest that the presence of both coding-SRA RNA and its corresponding SRAP modifies the activity of the estrogen receptor in breast cancer cells and that SRAP could be a new clinical marker for breast cancer. Further studies are needed to

define the respective mechanisms of action and the roles of SRA RNA and protein in breast tumorigenesis and tumor progression.

Introduction

Through its action on breast epithelial cells, estrogen not only controls the growth and the development of the normal mammary gland but also promotes breast tumorigenesis and breast cancer progression.[1] The biological action of estrogen is mainly mediated through two estrogen receptors (ERs), alpha and beta, which act as ligand–dependent transcription factors.[2;3] While estrogen initially plays a pivotal role in the activation of ERs, the transcriptional activation of target genes is ultimately determined by interactions between receptors and regulatory molecules known as co-activators and co-repressors, which respectively stimulate or inhibit ER activity.[4] The Steroid Receptor RNA Activator (SRA) differs from all previously characterized co-activators as it has originally been identified as a functional non-coding RNA molecule.[5] SRA mechanisms of action have since become the focus of extensive investigation. SRA was shown to contain a core RNA sequence necessary and sufficient to mediate steroid receptor activity [6] through interactions with several proteins including the co-activator/co-repressor SHARP (SMRT/HDAC Associated repressor protein) [7], the Steroid Receptor Co-activator 1 (SRC1) [5], and the AF-1 specific activator p72/p68 protein.[8] Post transcriptional modifications of SRA have also been shown to participate to the ability of this RNA to modulate receptor activity.[9] We were the first to establish that SRA RNA was differentially expressed in normal and in breast tumor tissue and to suggest that SRA RNA could be involved in mechanisms underlying breast tumorigenesis and breast tumor progression.[10] The recent observation by Lanz et al of multiple proliferation anomalies in the over-expressing non-coding SRA RNA mammary glands of transgenic mice corroborates this hypothesis. [11]

While all these studies refer to SRA as a non-coding RNA, we have recently demonstrated the existence of coding SRA RNA isoforms and corresponding endogenous SRA proteins [12], highly conserved in vertebrates and expressed in breast cancer cell lines. [13;14] To date, no data is available on the possible role of the coding-SRA RNA or SRAP in breast cancer cells or on their expression in human breast tumor tissues. Here, we investigated SRAP expression in a cohort of ER positive primary breast tumors from patients subsequently treated with tamoxifen and examined the effect of SRAP over-expression on ERα activity in MCF-7 mammary tumor cells.

Materials and Methods.

Human breast tissues and cell lines.

Seventy four primary breast tumors were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Tumors corresponded to cases associated with node-negative status, that were treated by surgery with or without radiation therapy and then tamoxifen endocrine therapy. All tumors were ER positive (ER levels ranging from 4 to 247 fmol/mg protein, median 45.5) and spanned a wide range of Progesterone Receptor levels (PR levels ranging from 2.4 to 444 fmol/mg protein, median 31). Notthingham grade was known for 66 tumors, which were assigned to low (n = 23, scores 3-5), moderate (n = 35, scores 6-7) or high (n = 8, scores 8-9) categories. MCF7 were stably transfected with the pCDNA.3.1-V5-His expression vector alone (Control cell line) or containing a coding- SRA cDNA to generate the SRAP-V5 high A, SRAP-V5 high B and SRAP-V5 low cells lines as previously reported.[14]

Western blot analysis.

Total proteins were extracted from cells [14] or breast tumor tissues [15] and analyzed by Western blot as previously described.[13-15] Four primary antibodies, a rabbit polyclonal anti-

SRAP antibody[14], a mouse monoclonal anti-ER antibody (NCL-ER 6F11/2, Novocastra, Newcastle, UK), a mouse monoclonal anti-PR antibody (NCL-PGR 312, Novocastra) and a mouse monoclonal anti-β-actin antibody (A5441, Sigma, Oakville, ON) were used in this study at a dilution of 1:1000, 1:1000, 1:1000 and 1:5000 respectively. Pre-incubation of the primary anti-SRAP with its corresponding peptide was performed as described previously.[14]

To examine PR expression, MCF-7 cont, SRAP-V5 high A, SRAP-V5 high B and SRAP-V5 low were grown for 6 days in serum free phenol red free DMEM supplemented with apotransferin, BSA . Cells were then treated with either ethanol (vehicle) or estradiol (10^{-8}M) for 4, 24 and 48 hours. Cells were then lysed , and identical amounts of total protein extracts were analysed as described above. To ensure equal loading gels were stained with coomassie blue.

RT-PCR analysis.

MCF-7 cont, SRAP-V5 high A, SRAP-V5 high B and SRAP-V5 low cells were grown for 6 days in serum free phenol red free DMEM supplemented with apotransferin, BSA and subsequently treated with either ethanol (vehicle), estradiol (10^{-8}M) for 15 and 60 minutes. Total RNA was isolated from these cells using the eppendorf RNA isolation kit (Eppendorf). cDNA was synthesized using MMLV reverse transcriptase (Invitrogen) as described in the manufacturer's instructions. cDNAs were then amplified with platinum taq polymerase (Invitrogen) as previously described. [16] The primers to TFF1 were upper 5′-CTGGGGCACCTTGCATTTTCC-3′ and lower 5'-CGGGGGGCCACTGTACACGTC-3'[17] and to GAPDH were upper 5'-ACCCACTCCTCCACCTGG-3' and lower 5'-CTCTTGTGCTCTTGCTGGG-3'.

The PCR products were separated electrophoretically on a 2 % agarose gels subsequently stained with ethidium bromide. The gels were visualized under UV light on a GelDoc2000/ChemiDoc System (BioRad).

Cell transfection experiments.

Transfection experiments were performed as previously described [18] with small modifications. Briefly, cells grown in phenol red free DMEM supplemented with 5% charcoal stripped FBS for 48 hours, were transfected for 4 hours with 1μg of ERE-luciferase plasmid (containing a vitellogenin ERE "GGTCACTGTGACC" site upstream of the firefly luciferase cDNA) and 0.1 μg of Renilla luciferase reporter vector (Promega, Madison, WI) using the Lipofectamine reagent (Invitrogen, Burlington, ON). Cells were then treated with either ethanol (vehicle), estradiol (10^{-6}M to 10^{-10} M) and/or 4-hydroxy-tamoxifen (Sigma) (10^{-6}M to 10^{-9}M) for 24 hours. Cells were lysed in 200 μl of cell culture lysis buffer (Promega) and lysates analyzed for luciferase and renilla luciferase activities according to the manufacturer's protocol (Promega).

Statistical Analysis.

Transfection results were normalized by dividing the measured ERE luciferase activities by their corresponding renilla luciferase activities. For each treatment, the relative luciferase activity was then used to calculate fold induction (ratio of value for a treatment and the corresponding value for ethanol treatment). Results are representative of at least 3 independent experiments. Significant differences were assessed using the Student's T test. Error bars represent standart error of the mean (SEM).

Tumor cases were classified as SRAP positive and SRAP negative subgroups following the independent assessment of the corresponding Western blot signal by two investigators.

Statistical differences in ER/PR or Nottingham grade between the two groups were tested using the Mann-Whitney test (two-tailed) or the Chi square test, respectively. Relapse-free survival was defined as the time from initial surgery to the date of clinically documented local or distant disease recurrence or death attributed to breast cancer. Overall survival was defined by the time from initial surgery to the date of death attributed to breast cancer. Deaths caused by other known or unknown causes were censored. The association between SRAP expression and relapse or survival was assessed by the Kaplan-Meier method.

Results

Differential expression of SRA protein in breast tumor tissues.

To investigate the possible relationship between SRAP expression and known prognostic markers, we performed Western blot analysis on a series of protein extracted from 74 different ER positive breast tumors. A strong background signal (50 kDa), still present with the neutralizing peptide, was observed in all tumors (Fig.1A-B).

SRAP signal, which disappeared when the antibody was pre-incubated with the corresponding peptide, was observed in some (24 tumors such as lanes 1, 2, 8) but not all (50 tumors, lanes 3, 4, 5, 7) tumors (Fig.1A-B). As expected, the size of the bands observed varied around 30 kDa, likely as a result of the genetic background (homozygous/heterozygous for the different alleles) of the patients [14]. It should be noted that a band of ~25 kDa, not previously seen in breast cancer cell lines [14], was also specifically recognized in 34 cases (see lane 1, 2, 4-8, Fig.1A).

Neither ER nor PR levels were significantly different (Mann-Whitney rank sum test, $p>0.05$) between SRAP positive (n=24, median ER=74.5 & PR=30.5 fmole/mg protein) and SRAP negative (n=50, median ER=39 & PR=32.5 fmole/mg protein) subgroups. Similarly no

significant relationship (Chi square test, p> 0.05) was found between SRAP expression and histological tumor grade distribution (Low grade: n = 7 & n=16, Moderate grade: n=9 & n= 26, High grade: n= 5 & n= 3 for SRAP positive and negative subgroup, respectively).

When SRAP expression was considered in relation to the proportion of recurrence, no significant difference was seen (number of events: 9 for SRAP negative patients, and 3 for SRAP positive patients, Fig.1C). In contrast, when considered in relation to outcome (Fig. 1D), a significant (Kaplan Meier, p=0.044) association was found with undetectable level of SRAP expression and poor survival (number of events: 7 for SRAP negative and 0 for SRAP positive group, respectively).

No correlation was found between the expression of the 25 kDa band and any tumor or patient characteristics (data not shown).

Obtention of clones stably expressing SRAP-V5 recombinant protein.

We stably transfected MCF-7 mammary cancer cells, known to express high level of endogenous ER, with a construction consisting of coding-SRA RNA able to encode a fusion protein SRAP-V5-tag. [14] Several clones were selected and protein extracts analyzed by Western blot using an anti-SRA antibody previously shown to recognize both endogenous SRAP (~30 kDa) and V5-tagged SRAP protein (~35 kDa).[14] As shown on Fig.2 A, clones expressing detectable levels (SRAP-V5-High.A and SRAP-V5-High.B) or not detectable levels (SRAP-V5-Low) of the recombinant SRAP-V5 protein were obtained. All cell lines, including the control MCF-7 stably transfected with the vector alone, expressed identical levels of endogenous SRAP (Fig.2A), ER (Fig.2B) and Progesterone Receptor isoforms A (112 kDa) and isoform B (83 kDa) (Fig.2 C), as assessed by Western blot analysis.

Decreased ligand dependent transcriptional activity of ER in SRAP-V5 over-expressing cells

To establish whether the ER signaling pathway was altered in cells over-expressing SRAP, an ERE-luciferase reporter vector was transiently transfected in all four cell lines described above. Cells were then treated with ethanol (vehicle), estradiol (10^{-8} M) or 4-hydroxy-tamoxifen (10^{-6} M) for 24 hours and luciferase activity measured, as described in the Materials and Methods section. Luciferase activities of the four cell lines were identical when cells were treated with ethanol or 4-hydroxy-tamoxifen (data not shown and Fig.3A). However, upon estradiol (10^{-8} M) treatment, cells over-expressing SRAP-V5 (SRAP-V5-High.A and SRAP-V5-High.B) showed a significant lower induction (~19 folds) (Student's T test, $p < 0.03$) of ERα transcriptional activity compared to the induction observed in control cells (~39 fold, Fig.3A). In contrast, even though the reporter gene induction was slightly lower (~32 folds) in SRAP-V5-Low cells compared to that in control cells (~39 fold), this difference did not reach statistical significance ($p>0.05$).

To establish whether the decreased activation of ER in SRAP-V5 over-expressing cells was dependent on the dose of estradiol used, we transiently transfected all four cell lines with and ERE reporter gene and treated the cell lines with increasing amount of estradiol (10^{-6} M to 10^{-10} M E2). A lower activation of the reporter gene in SRAP-V5 over-expressing cells compared to control cells (Fig.3B) was observed for all concentrations of estradiol used. Differences between the SRAP over-expressing and control cell lines were statistically significant ($p<0.02$) at 10^{-8} M, 10^{-9} M and 10^{-10} M E2. We suspect treatments with higher E2 concentrations (10^{-6} M and 10^{-7} M) of having toxic effects on cells that could have lead to a lower reproducibility between experiments.

Similar tamoxifen sensitivity in SRAP-V5 over-expressing and control cell lines

To determine whether SRAP over-espression potentiates the inhibitory effect of 4-hydroxy-tamoxifen, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells transiently transfected with an ERE-luciferase reporter vector were treated with 10^{-8} M estradiol supplemented with increasing amounts of 4-hydroxy-tamoxifen (10^{-9} M to 10^{-6} M). The reporter gene activities were lower in SRAP over-expressing cells compared to the activities observed for the corresponding treatment in control cells (Fig. 3C). However, in all cell lines, the first efficient concentration of 4-hydroxy-tamoxifen able to significantly ($p<0.05$) decrease E2 dependent induction was 10^{-7} M (Fig.3C).

Higher PR expression upon estradiol stimulation in SRAP-V5 over-expressing cells

It was important to determine whether the difference in the response to estradiol observed between high and low SRAP expressing MCF-7 cells seen with the luciferase reporter assay could also be observed during the induction of known ER target genes. In order to address this question, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells were grown in serum free media and subsequently treated with 10^{-8} M estradiol for 4 hours, 24 hours and 48 hours. Proteins were extracted and identical amounts of total protein lysates were analyzed by Western Blot for the PR expression as described in the Materials and Methods section. As shown in Fig.4A, PR protein was extremely low or even undetectable in all four cell lines when grown in serum free media for 6 days (t= 0) and when treated with vehicle alone. Interestingly, upon 24 hours and 48 hours of estradiol stimulation, SRAP-V5 High.A, High.B expressed noticeably higher PR levels than the control [19] cell line. To ensure equal loading, SDS-Page gels were stained with coomassie blue and the intensity of the staining was shown to be identical in all cell lines (data not shown). As seen in Fig. 4A, the SRAP-V5 Low cell

line also had a higher PR induction at 24 and 48 hours than the control cell line. However the PR expression was considerably lower in the SRAP-V5 Low cell line compared to the PR expression seen in SRAP-V5 High.A, High.B cell lines at 48 hours.

Similar etsradiol dependent TFF1 mRNA induction in SRAP-V5 over-expressing and control cells.

Trefoil factor 1 (TFF1) is another well characterized ER target gene which expression was found increased by estradiol as early as 1 hour of treatment MCF-7 cells [20]. In order to determine whether the estradiol dependent induction of TFF1 is differentially regulated in SRAP over-expressing cells, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and Cont cells were grown in serum free media and subsequently treated with 10^{-8} M estradiol for 15 and 60 minutes. Total RNA was extracted, reverse transcribed and analyzed by RT-PCR using primers recognizing TFF1 cDNA as described in the Materials and Methods section. As shown in Fig. 4B, a similar increase in TFF1 mRNA levels was observed in all four cell lines upon 60 minute 10^{-8}M estradiol treatment.

Discussion

To date, all functional studies on SRA only focus on its RNA aspect and were performed in transient expression systems. This study is the first to establish the existence of the corresponding SRAP protein in breast tumor tissues and to examine the possible implication of SRAP expression on the ER signaling pathway.

SRAP was detected by Western blot analysis in 24 out of 74 (32%) cases, migrating at around 30 kDa and appearing either as a single band or doublet. We suspect that the diverse band pattern observed in breast tumor tissues results from the different genetic background of the patients. Indeed, we have previously demonstrated the existence of three SRAP isoforms with

SRA isoform 3 migrating slightly slower than the two other isoforms.[14] The differences in the SRAP migration in the tumor samples could therefore be due to homozygosity/heterozygosity for the different SRA isoforms and/or the differential use of either the first or second methionine as described previously. [13]

In addition to the expected 30 kDa band, we also observed in 34 out of 74 cases (45%) a 25 kDa band specifically recognized by our antibody. Although the theoretical size of SRAP is 25 kDa, we had previously never detected a 25 kDa SRAP in any of the breast cancer cell lines grown in vitro and analyzed by Western blot. [14] This new form of SRAP seen in breast tumor tissues can not be attributed to an alternative translation starting at the second methionine as a 12 amino acids difference would not account for a shift in migration by 5 kDa. Similarly, the 25 kDa can neither correspond to an alternative translation starting at the third methionine (at amino acid poosition 75) since this form of SRAP would not be detected by our antibody targeted against amino acids 20 to 34. We suspect this 25 Kda band to SRAP with distinct post-translational modifications from the ones observed on the 30 kDa SRAP. Whether both SRAP are expressed by the same cells remains to be determined. Antibodies directed against different regions of SRAP will be generated to address these issues.

No significant correlations were found between the detection of any SRAPs (30 kDa or 25 kDa) and the levels of ER, PR, or grade of the tumors. Similarly, no correlation was observed between the reoccurrence of the disease and SRAP expression. We have however found that the expression of the 30 kDa SRAP correlated with an overall better survival in ER positive patients subsequently treated with tamoxifen. This suggests that SRAP could be a new independent prognostic marker that might predict the outcome of the disease. In other words, the detection of SRAP in the primary tumor could be a marker of a “less aggressive” form of

cancer. Further analyses, performed on larger cohorts of patients associated with different tumor subgroups, are needed to corroborate this hypothesis.

The observed correlation between SRAP expression and an overall better survival in ER positive patients prompt us to examine further the impact of the coding SRA RNA and consequently SRAP on the ER signaling pathway. Here, we show that the over-expression of SRAP protein in breast cancer MCF-7 cells results in a decreased responsiveness to estrogen (for all concentrations used), as assessed by activation of a transiently expressed ERE-luciferase reporter gene. This decrease cannot be attributed to a change in ER-α expression since similar levels of this receptor are detected in the control and SRAP over-expressing cells. SRA RNA over-expression had previously been shown to potentiate ER and PR transcriptional activities.[5;18] It was therefore unexpected to observe a decreased ER activity in cells stably over-expressing SRA RNA. To our knowledge, all the SRA sequences used by others when investigating SRA RNA function lacked the first two starting methionines and were consequently unable to encode the SRAP protein.[5-7;11] The SRA sequence used here contains 32 additional 5' end base pairs with two putative starting methionines and therefore has the capacity to initiate the translation of either a 236 or 224 amino acids SRAP.[14] This coding RNA is expected to function as an ER activating RNA since it contains an intact SRA core sequence previously shown to be necessary and sufficient for SRA RNA to function as a non-coding RNA.[5] Our luciferase reporter assays therefore indicate that the concurrent coding-SRA RNA and protein expressions result in a significant reduction of ER activity. We are the first to observe such opposite action of the coding SRA RNA from the previously reported steroid receptor co-activating function of the non-coding SRA RNA. We therefore suspect that SRAP expression to be responsible for this apparent lower ER activity. Further

studies are however needed to demonstrate this hypothesis. In addition, our results suggest that the concurrent expression of coding-SRA RNA / SRAP, selectively modifies the activity of estradiol stimulated ER, but does not affect the ER sensitivity to tamoxifen. This fits the observation that patients whose primary tumors expressed SRAP did not have a lower incidence in reoccurrence of the disease. Indeed, in the light of our reporter gene assay, tumor cells expressing high levels of SRAP are not suspected to respond better to tamoxifen.

Similar PR levels were observed in the control and SRAP over-expressing cells when cells were grown in complete media. PR is an ER target gene and an apparent decrease in ER activity, through over-expression of SRAP, was expected to lead to lower PR levels in these cells. Surprisingly however, when cells were grown in serum free media and subsequently treated with estradiol, PR expression was induced faster in the SRAP-V5 over-expressing cells. Although non-coding SRA RNA has previously been shown to increase PR expression and activity [5], it is premature today to attribute the increased PR levels to either the expression of the RNA or protein. Indeed our MCF-7 cell model reflects a more complex system with concurrent actions of both SRA RNA and protein. This model, although more comprehensive, does not allow the separation and analyses of the SRA RNA and protein functions. In addition, it is now increasingly apparent that co-regulating molecules altering ER activity do not have a global effect but rather distinct outcomes on individual target genes [21]. In support to this concept, we observed that the estradiol mediated regulation of the two ER target genes studied (PR and TFF1) was dissimilar in the SRAP-V5 over-expressing and control cells. In fact, while the estradiol mediated induction of the PR was evidently distinct between the SRAP over-expressing and control cell lines, no difference in TFF1 induction was observed between the cells. Furthermore, although non-coding SRA RNA has been shown to act as estrogen receptor

activator, a recent study has shown that it is only able to activate distinct ER target gene promoters [22]. Additional studies are needed and will be performed to dissect separately the exact mechanisms of action of the SRAP protein and SRA RNA and subsequently analyze their respective action on individual ER target genes. Nonetheless our reporter assays and analysis of PR expression have demonstrated that expression of the coding SRA RNA leads to an alteration in the ER signaling pathway distinct from the previously reported effect of the non-coding SRA RNA.

To date, all functional studies on SRA only focus on its RNA aspect. This study is the first to establish the existence of the corresponding SRAP protein in breast tumor tissues and to examine the possible implication of a coding SRA RNA and consequently SRAP expression on the ER signaling pathway and breast cancer progression. The discovery that SRAP might itself also be implicated in the ER signaling pathway and its expression correlates with disease outcome emphasizes the need to actively probe into the exact mechanisms of action of this increasingly complex but promising bi-faceted molecule. Indeed, additional studies, examining the separate and concurrent functions and regulations of SRA RNA and SRAP protein, are essential to establish the clinical potential of these bi-faced molecules in the treatment of breast cancer.

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22. KLINGE, C.M., JERNIGAN, S.C., MATTINGLY, K.A., RISINGER, K.E., and ZHANG, J., Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. *J.Mol.Endocrinol.*, **33**, 387-410, (2004).

Figure Legends

Fig.1. SRAP expression correlates with overall better survival in ER+ breast cancer patients. Proteins were extracted from a cohort of 74 ER positive tumors and analyzed by Western blot for SRAP expression as described in the Material and Methods section. (**A**) Representative panel showing Western blot for tumors 1 to 8. C: SRAP-V5-High.A cells. (**B)** Western blot performed in parallel and incubated with an anti-SRA antibody pre-mixed with the neutralizing peptide. **(C)** and **(D)** Kaplan-Meier graphs for "Time to progression" and "Overall survival" with respect to SRAP expression.

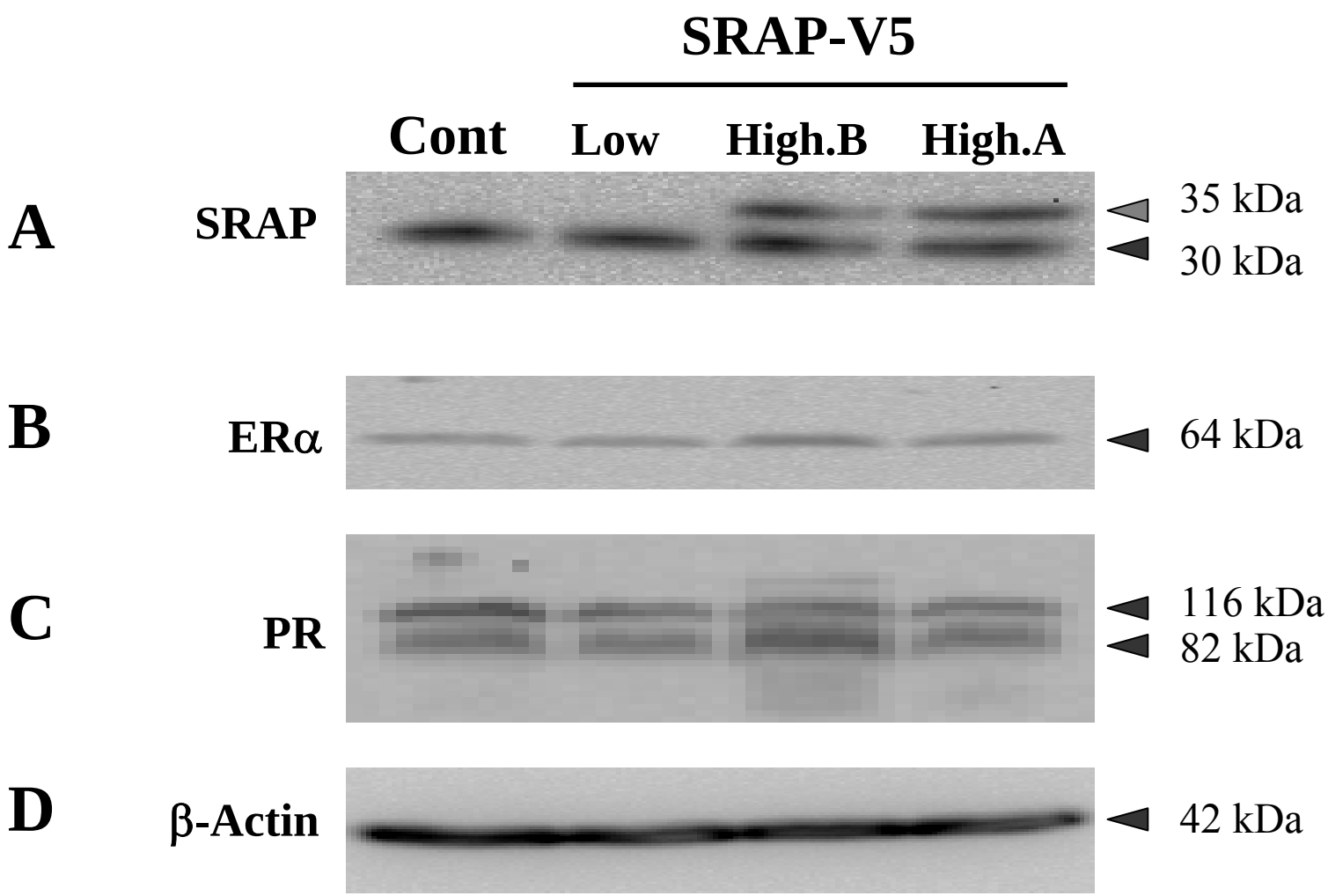
Fig.2. Western blots analysis of MCF-7 cells stably transfected with SRAP-V5 cDNA. MCF-7 breast cancer cells were stably transfected with SRAP-V5 cDNA (SRAP-V5) or empty vector and total protein extracts analyzed by Western blot as described in the Materials and Methods section. Antibodies used consisted of anti-SRAP **(A)**, anti-ER-α **(B)**, anti-PR **(C)** and anti-actin **(D)**. Two high (High.A and B) and one not detectable (Low.A) SRAP-V5 expressors were selected for further analysis.

Fig.3. Decreased activation of an ERE-luciferase reporter gene in SRAP-V5 over-expressing cells. MCF-7 cells expressing detectable (SRAP-V5 High.A and B) or not detectable levels (SRAP-V5 Low and Cont) of SRAP-V5 recombinant protein were transiently transfected with an ERE-luciferase reporter gene and subsequently treated with **(A)** ethanol, 10^{-8} M E_2, or 10^{-6} M of 4-hydroxy-tamoxifen **(B)** 10^{-6} M to 10^{-10} M E_2 or **(C)** 10^{-8} M E2 + 0, 10^{-9} M (-9), 10^{-8} M (-8), 10^{-7} M (-7), 10^{-6} M (-6) of 4-hydoxy-tamoxifen for 24 hours. The results correspond to the average of fold induction (ratio between luciferase values during

ligand treatment and the corresponding ethanol treatment) of at least three independent experiments. Bars: Standard error of the mean (SEM). Cont: MCF-7 cells stably transfected with vector alone. * represents for **(A)** and **(B)** a statistical significant difference ($p<0.05$, Student's test) between the fold induction obtained for SRAP-V5 over-expressing clones and the corresponding fold induction for the control cells. For **(C)** * represents the fold inductions for specific tamoxifen concentrations that were statistically lower ($p<0.05$, Student's test) from the corresponding fold induction at 10^{-8} M estradiol for each cell line.

Fig.4. SRAP-V5 over-expressing cells have a higher PR but not TFF1 expression upon estradiol stimulation. . MCF-7 cells expressing detectable (SRAP-V5 High.A and B) or not detectable levels (SRAP-V5 Low and Cont) of SRAP-V5 recombinant protein were grown in serum free media for 6 days and subsequently treated with ethanol or 10^{-8} M E_2, for **(A)** 4, 24 and 48 hours or **(B)** 15, 60 minutes as described in the Materials and Methods section. **(A)** Cells were lysed and identical amounts of total protein extracts were analyzed by Western Blot for PR expression. Identical amount (100 μg) of a lysate positive for PR expression was used as positive control (c). Black arrows indicate PR isoform A (112 kDa), and the grey arrow indicate PR isoform B (82 kDa). **(B)** Cells were lyzed and extracted RNAs were reversed transcribed and then analysed for TFF, and GAPDH expression by PCR as indicated in the Materials and Methods section.

Fig.1


SRAP-V5
Cont
Low
High.B
High.A
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SRAP
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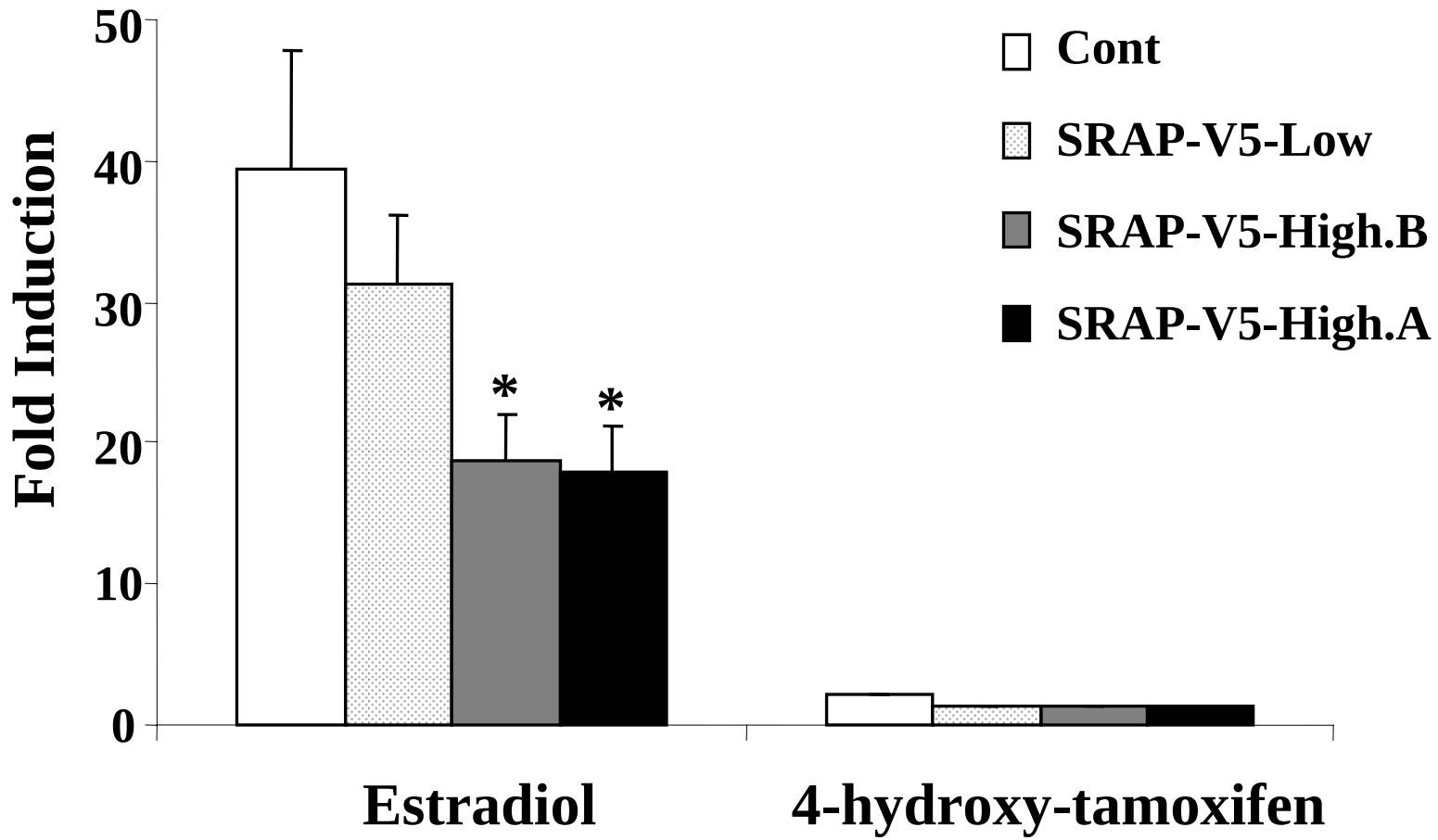
30 kDa
B
ERα
64 kDa
C
PR
116 kDa
82 kDa
D
β-Actin
42 kDa

Fig.2


Fold Induction
50
40
30
20
10
0
Cont
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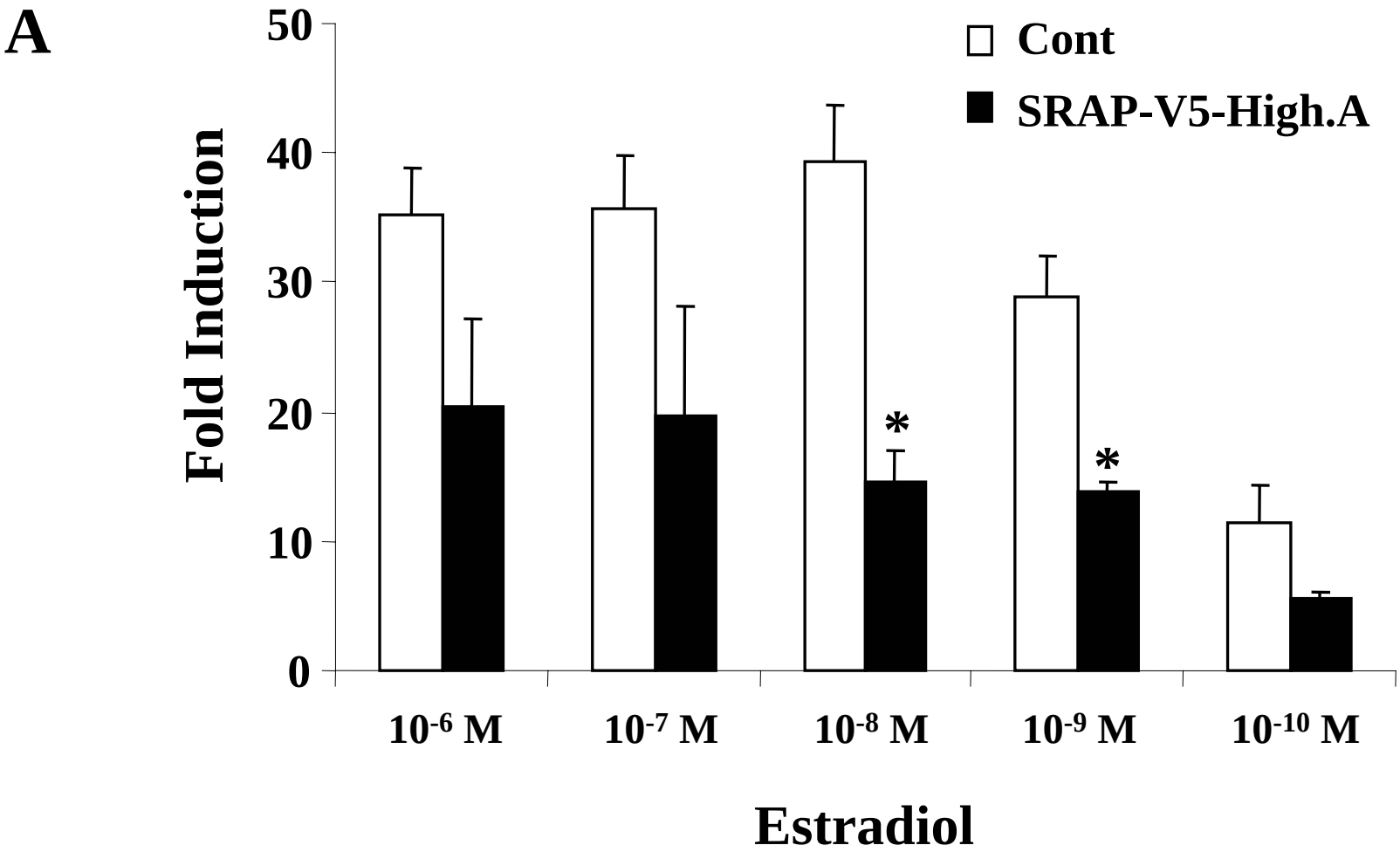
SRAP-V5-High.B
SRAP-V5-High.A
*
*
Estradiol
4-hydroxy-tamoxifen

Fig.3


A
Cont
SRAP-V5-High.A
Fold Induction
50
40
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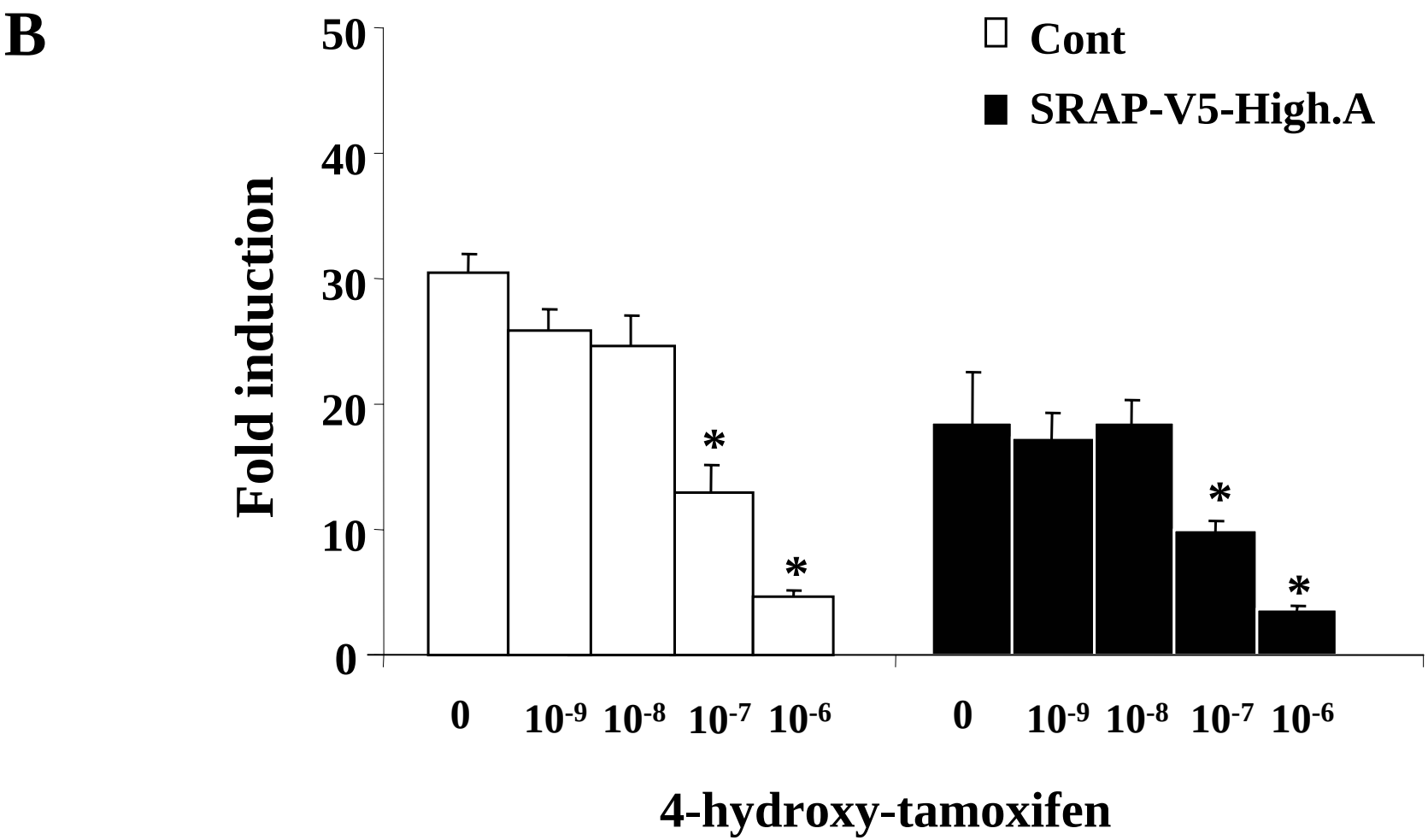
*
*
10-6 M
10-7 M
10-8 M
10-9 M
10-10 M
Estradiol

Fig.3


B
Cont
SRAP-V5-High.A
Fold induction
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4-hydroxy-tamoxifen

Fig.4

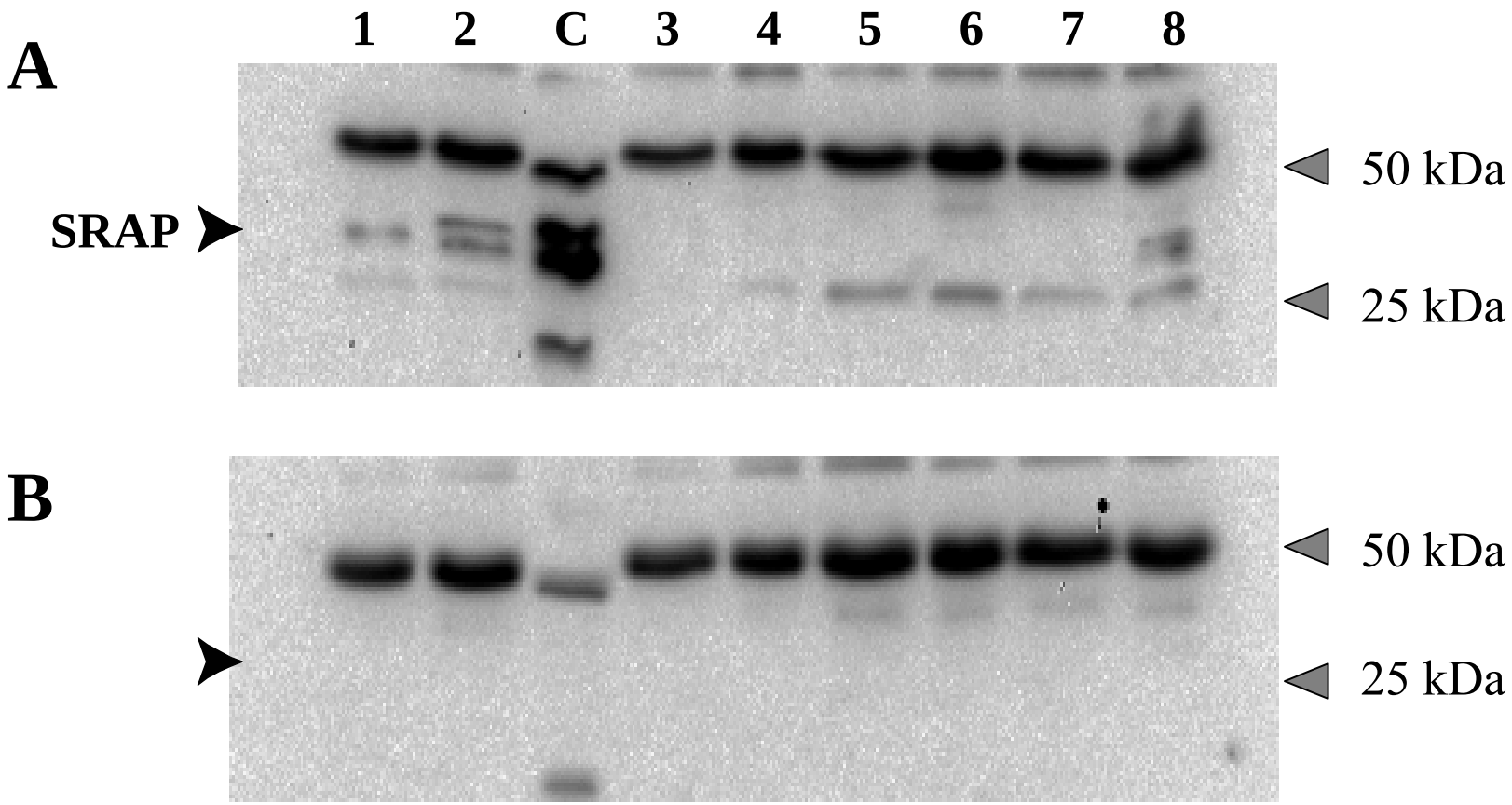


Fig.4

C

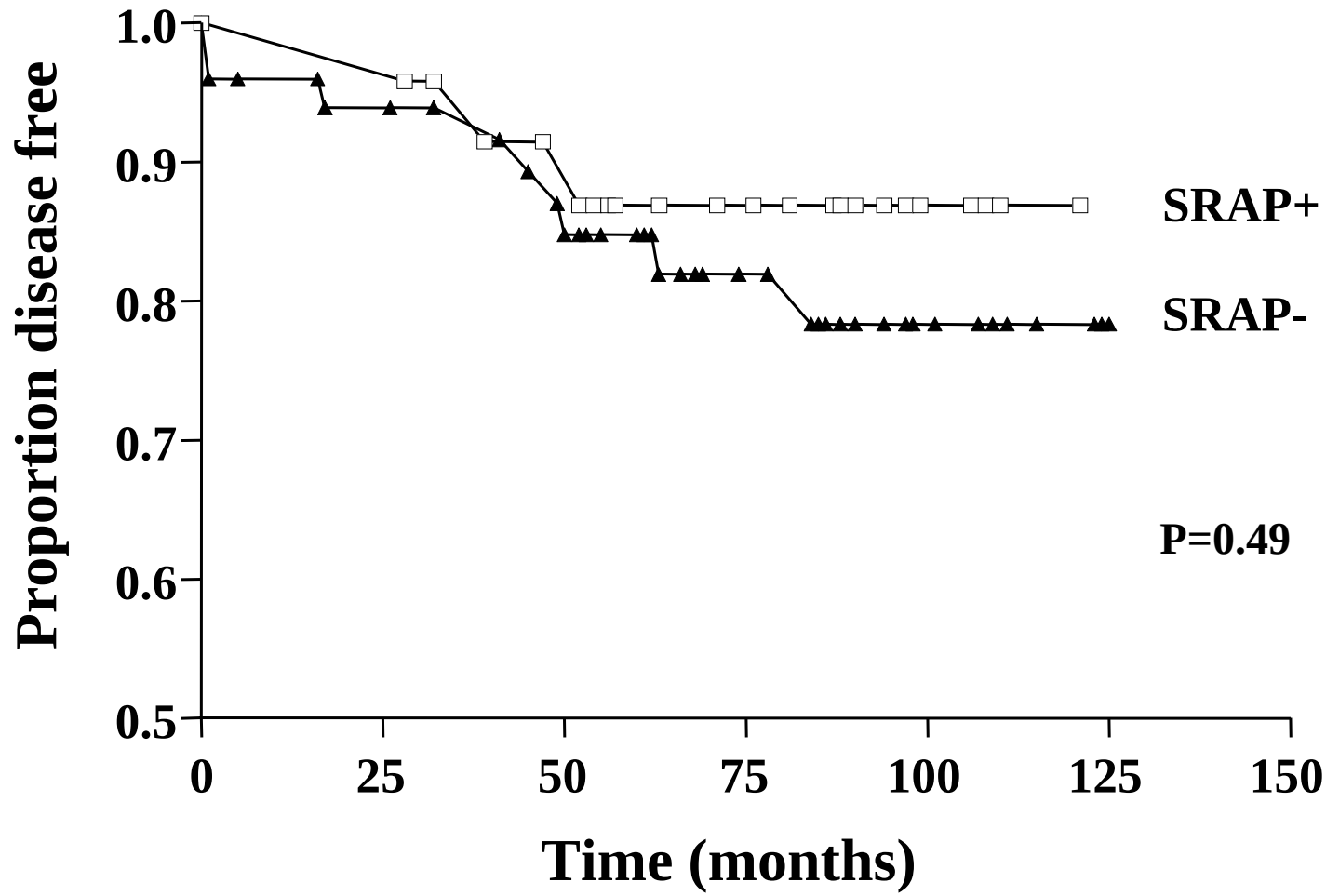
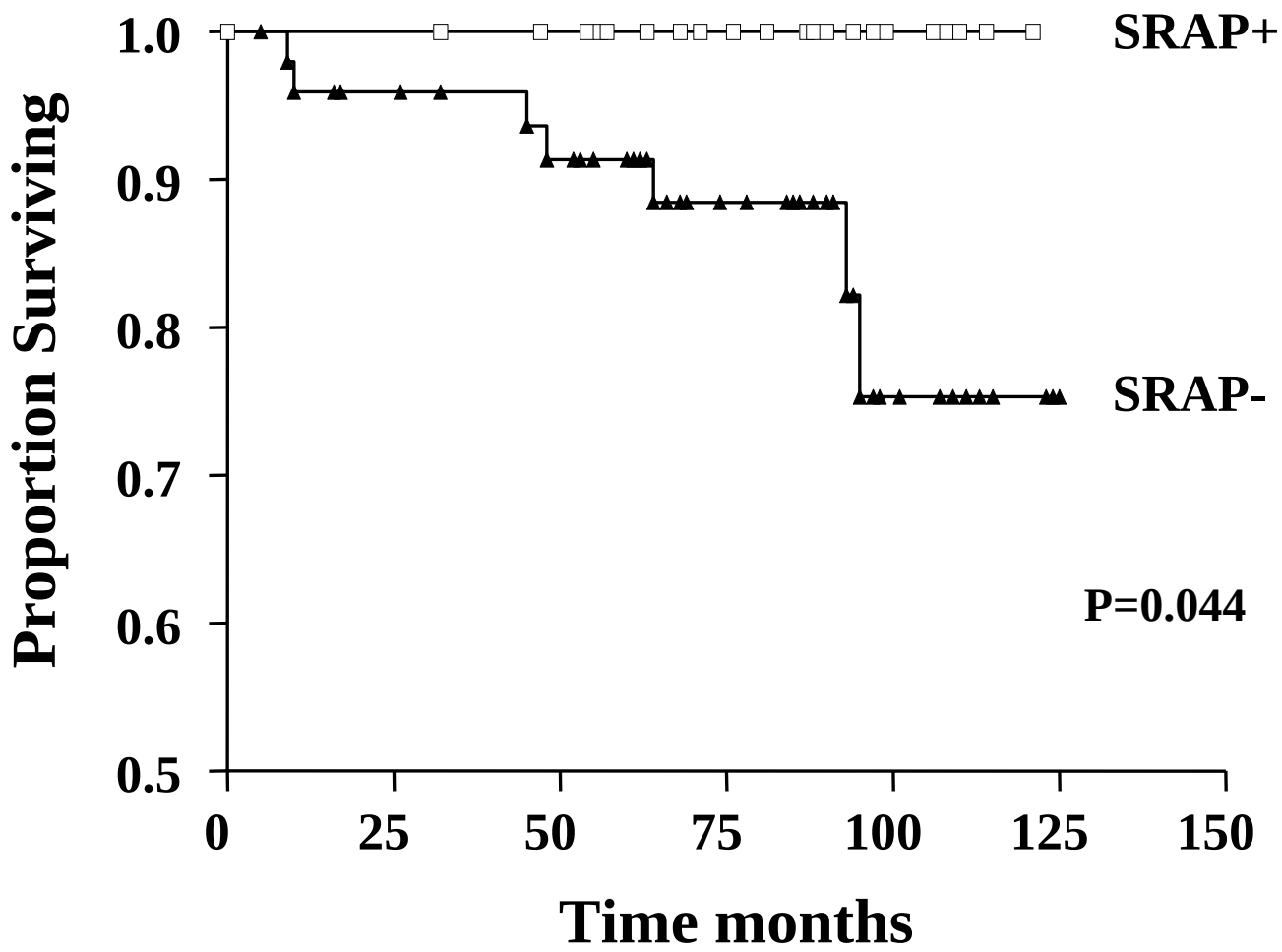

1.0
0.9
0.8
0.7
0.6
0.5
Proportion disease free
0
25
50
75
100
125
150
Time (months)
SRAP+
SRAP-
P=0.49

Fig.4

D


1.0
0.9
0.8
0.7
0.6
0.5
Proportion Surviving
0
25
50
75
100
125
150
Time months
SRAP+
SRAP-
P=0.044

Appendix 2

The Steroid Receptor RNA Activator Protein (SRAP) possesses an intrinsic transcription repressive activity.

Chooniedass-Kothari S, Hamedani M, Caracossa S, Jalaguier S, Cavailles V, Leygue E.

The steroid receptor RNA activator (SRA) differs from all previously characterized co-activators as it is the first and only example of a functional RNA able to encode a protein (SRAP). To date, all functional data on human SRA focus only on its non-coding RNA form. We have however recently reported that MCF-7 cells stably over-expressing SRAP have a significant decreased sensitivity to estradiol but no additional sensitivity to Tamoxifen in an ER luciferase reporter assay. We have also established that ER positive breast cancer patients whose primary tumors were positive for SRAP expression had a significant lower likelihood to die from recurrent disease than SRAP negative patients. In order to further understand SRAP mechanism of action, we have identified several putative SRAP interacting proteins by mass spectrometric analysis of co-immunoprecipitated samples. Several of these proteins, such as KAP-1 and MBD3, are involved in transcription regulation. In light of this data, we analyzed SRAP potential to modulate transcription. We have found that fusing SRAP to a gal4-VP16 chimeric activator protein lowers VP16 transcriptional activation, interestingly this effect is not observed when co-treating cells with trichostatin A (a Histone deacetylase inhibitor). Altogether, our data suggest that SRAP possesses an intrinsic transcriptional repressive activity possibly involving HDACs.
Grant Sponsors: CBCRI 12326 and USAMRMC W81XWH-05-1-0245.

Appendix 3

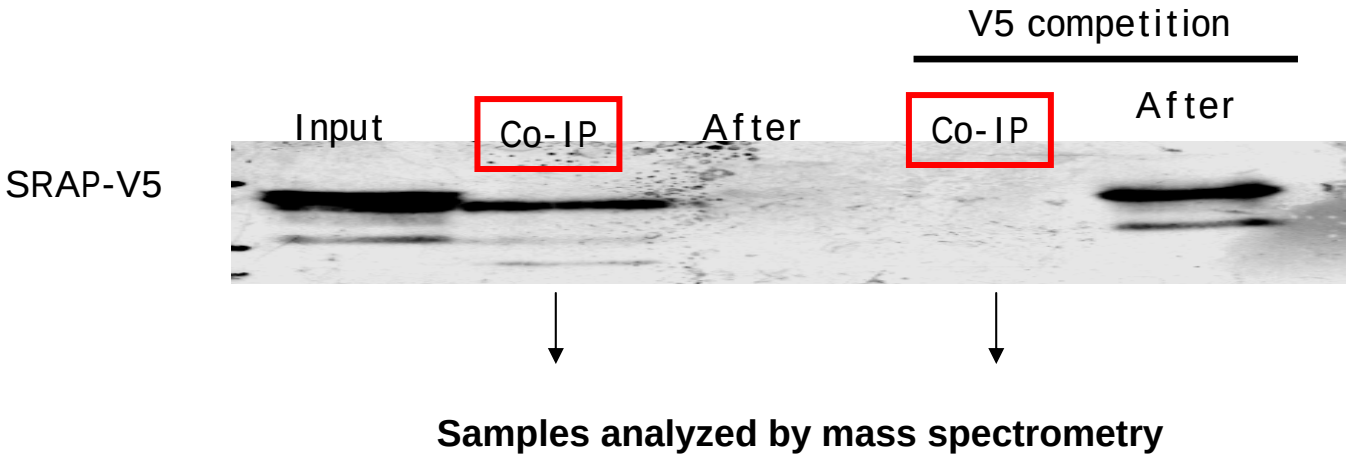


Figure 1: Western blot of SRAP co-immunoprecipitated with anti-V5 antibodies . Total protein extracts from MCF-7 cells stably expressing V5-tagged SRAP (input) were immunoprecipitated with anti-V5 antibodies in the absence or presence (V5 competition) of V5 peptide. Co-immunoprecipitated (CoIP) and remaining (after) fractions were checked by Western blot using anti-V5 antibodies. Both CoIP fractions were subsequently analyzed by Mass Spectrometry to identify SRAP interacting proteins.

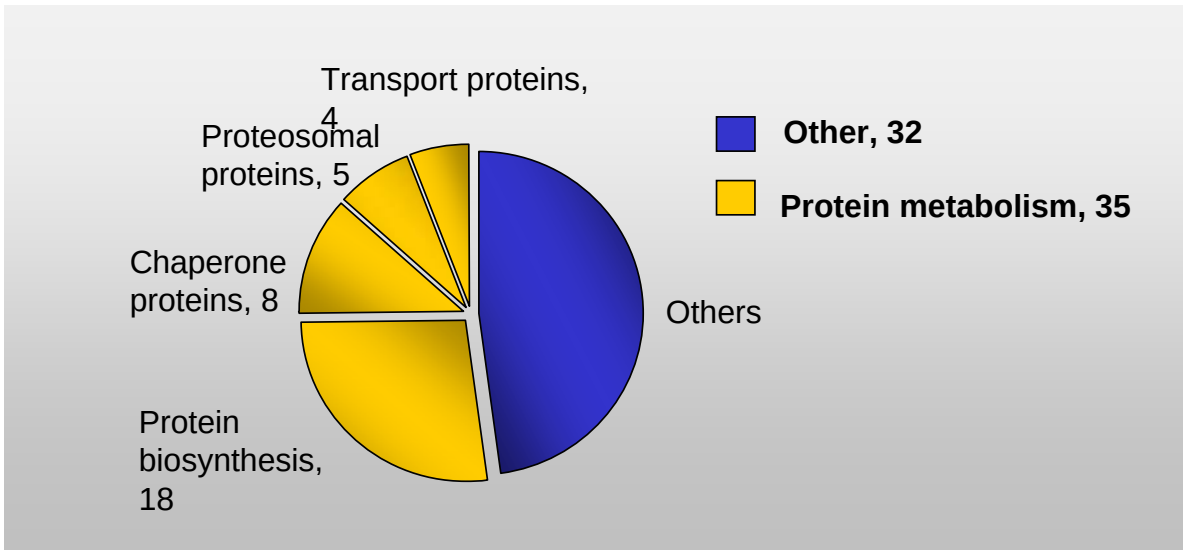


Figure 2. Functional grouping of SRAP interacting candidate proteins. The 67 proteins specifically immunoprecipitated with SRAP-V5 were grouped according to their general cellular function using the DAVID 2.1 software 4. In yellow are the proteins involved in general protein metabolism.

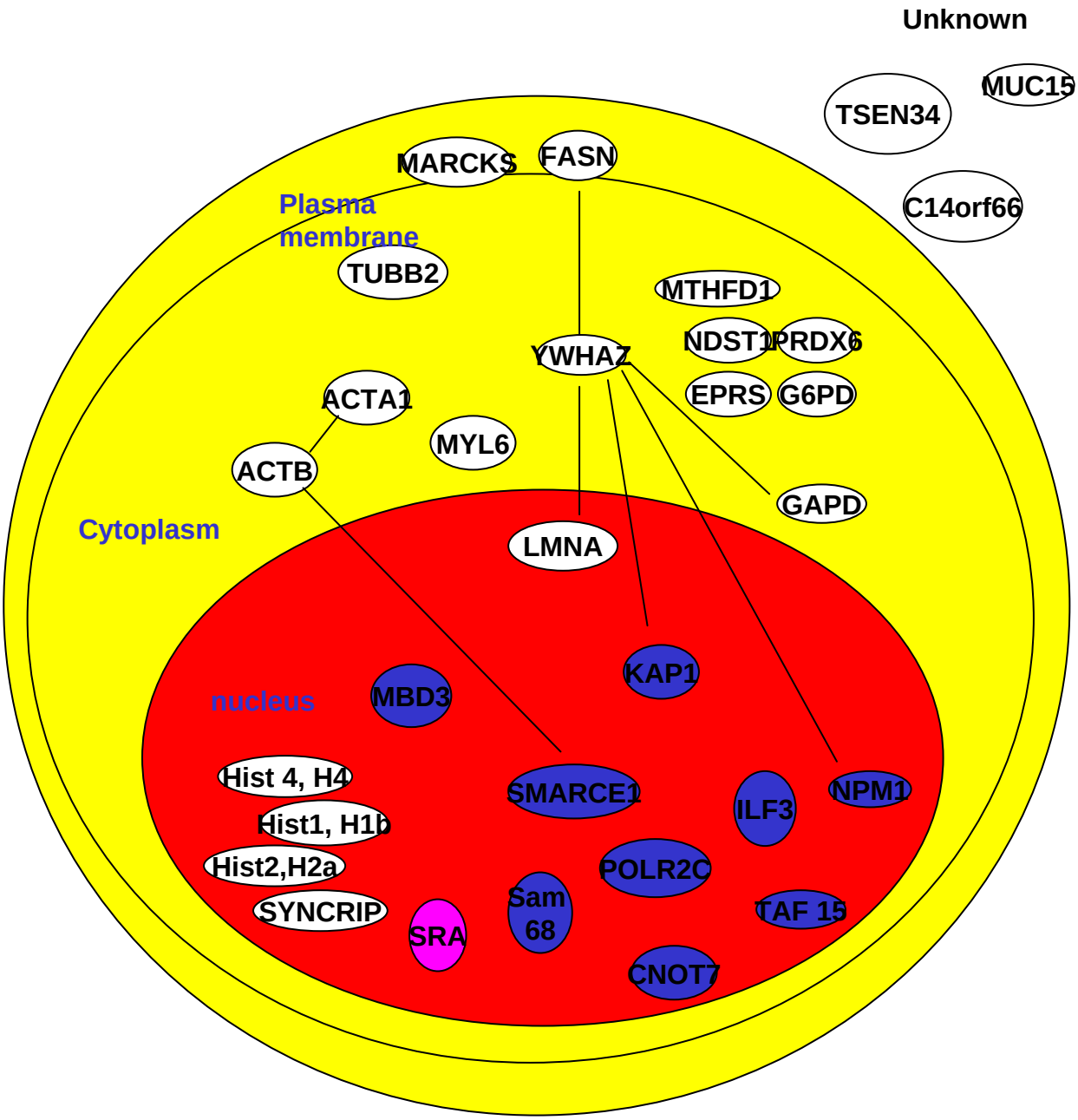


Figure 3: Cellular localization and known protein-protein interactions of the 32 proteins with functions other than general protein metabolism. Diagram was built with the Ingenuity Pathways Analysis 3.1. software 5. Proteins in purple are known to be involved in transcription regulation.